the residues are close to each other in closed, open or inactivated states. With this technique, we have detected and analyzed molecular movements within the voltage-sensor domain, between the voltage sensor and the pore domain, and within the pore domain.

Minisymposium 4: New Chemical Modulators by Rational Design

3927-MiniSymp
Optimizing Ligand-Protein Interactions Via Silcs: Site Identification by Ligand Competitive Saturation
Alexander MacKerell.
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Fragment-based methods for drug optimization have great potential; however, time, expense and sensitivity considerations associated with NMR and x-ray crystallographic based methods limit their applicability. As an alternative we have developed a computational approach, SILCS: Site Identification by Ligand Competitive Saturation, that uses explicit solvent all-atom molecular dynamics to identify binding sites on protein surfaces for functional groups. Information from the SILCS approach may then be combined with structural information on an inhibitor-protein complex to facilitate modification of the ligand to improve its binding affinity. An overview of SILCS and its application to inhibitor-ligand optimization will be presented.

3928-MiniSymp
Structure of P-Glycoprotein Reveals a Molecular Basis for Poly-Specific Drug Binding
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P-glycoprotein (Pgp) detoxifies cells by exporting hundreds of chemically unrelated toxins but causes multidrug resistance in the treatment of cancers. Substrate promiscuity is a hallmark of Pgp activity, thus a structural description of polyspecific drug-binding is vital for the rational design of anticancer drugs and MDR inhibitors. The x-ray structure of apo-Pgp at 3.8 Å reveals an internal cavity of ~6,000 Å3 with a 30 Å separation of the two nucleotide binding domains (NBD). Two additional Pgp structures with novel cyclic peptide inhibitors demonstrate distinct drug binding sites in the internal cavity capable of stereo-selectivity that is based on hydrophobic and aromatic interactions. Apo- and drug-bound Pgp structures have portals open to the cytoplasm and the inner leaflet of the lipid bilayer for drug entry. The inward-facing conformation represents an initial stage of the transport cycle that is competent for drug binding.

3929-MiniSymp
Chemical Synthesis of a Highly Selective Probe of the Renal Outer Medullary Potassium Channel (ROMK)
Jerod S. Denton, Brian A. Chauder, Rishin Kadakia, Eric S. Dawson, Craig W. Lindsley, C. David Weaver, Gautam Bhave.
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The renal outer medullary potassium channel, ROMK, critically regulates salt and water balance and may be a drug target for a novel class of diuretic. However, the molecular pharmacology of the inward rectifier potassium channel family is virtually undeveloped, precluding assessment of ROMK's therapeutic potential. We therefore performed a high-throughput screen of approximately 225,000 small molecules for modulators of ROMK function, from which several novel antagonists were identified. One compound, termed VU590, inhibits ROMK with a half-inhibition concentration (IC_{50}) of 300 nM, has no effect on Kir2.1 or Kir4.1, but inhibits Kir7.1 at low micromolar concentrations. Two structurally related compounds were identified in the screen, but were found to be comparatively weak ROMK inhibitors. Using a molecular mechanics-based knowledge of VU590, medicinal chemistry was employed to improve the potency of one compound 33-fold (IC_{50} from 8 μM to 240 nM). This novel probe, termed VU591, is highly selective for ROMK over Kir2.1, Kir2.3, Kir4.1, Kir6.2/SUR1B, Kir7.1 and a panel of more than 65 other potential off-targets, including voltage-gated sodium and calcium channels and hERG. Functional studies suggest the VU591 binding site is located in the cytoplasmic pore of ROMK. VU591 will be instrumental in mapping the location and topographical features of this selective binding site and could pave the way for animal studies assessing the therapeutic potential of ROMK.

3930-MiniSymp
Gold Nanoparticles as a Platform for Designing Protein-Protein Interaction Inhibitors: Application to Ubiquitin-Like Modifications
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Inhibitors of non-covalent protein-protein interactions hold much promise as useful probes to our understanding of human biology and disease mechanisms, as well as leads for developing new therapies. Developing such inhibitors, however, continues be a significant challenge. Protein-protein interactions mediated by ubiquitin-like (Ubl) modifications are among the most important signalling and regulatory mechanisms that control nearly every aspect of cellular functions. A unique feature of these post-translational modifications is the formation of poly-Ubl chains; however, strategies to target these poly-Ubl chain modified proteins are lacking. In this study, we show that gold nanparticles (AuNPs) conjugated with small molecule inhibitors can selectively target such poly-Ubl chains. Virtual ligand screening was carried out to identify small molecule mimmetics of the Small Ubiquitin-like MOdifier (SUMO) interaction motif in order to inhibit SUMO-mediated down-stream effects. Virtual ligand screening was based on the NMR structure of SUMO in complex with a peptide containing the SUMO-interaction motif. Interactions of the hit compounds with SUMO were investigated by NMR methods. One of the hits was modified for conjugation to an AuNP by adding a thiol tail. While the individual compounds do not have high affinity for SUMO (having K_d of 2 mM), conjugation of approximately 100 compounds to one AuNP allows for multi-valent interactions between AuNP and multiple SUMO proteins in a poly-SUMO chain; thus efficiently inhibits poly-SUMO-chain-mediated protein-protein interactions. This study demonstrates a viable approach to creating highly effective inhibitors by using AuNPs as a platform for multivalent interactions. This is the first application of AuNP for inhibiting Ubl modifications and provides a novel approach to specifically and effectively address such types of Ubl modifications for future research and therapeutic applications.

3931-MiniSymp
New Approaches to Anti-Infective and Anti-Cancer Therapeutics Targeting Metalloproteins
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I will give an account of recent progress in the development of novel anti-infectives targeting isoprenoid biosynthesis. Topics to be covered include: carotenoid biosynthesis as a target in staph and malaria; novel inhibitors of bacterial farnesyl and undecaprenyl diphosphate synthase; and the mechanism of action of the Fe4S4 cluster-containing proteins, GcpE (IspG) and LytB (IspH). We have investigated the mechanism of action and inhibition of Aquifex aeolicus LytB using a combination of site-directed mutagenesis (KM, Vmax), EPR and 1H, 2H, 13C, 31P and 57Fe-ENDOR. The EPR and ENDOR results support formation of an initial pi/sigma ''metallacycle'' complex similar to that observed previously with allyl alcohol bound to a nitrogenase FeMo cofactor. The complex is poised to interact with the E126 CO2H group, resulting in loss of H2O and formation of eta1 and/or eta3-allyl complexes. The IPP and DMAPP products are then formed in a second H+/e- reduction step. We also report that alkyne diphosphates are inhibitors of IspH and likewise form pi or pi/sigma metallacycle complexes, as evidenced by 1H, 2H, and 13C ENDOR. I will also give an update on the mechanism of action of GcpE, and the discovery of potent, mechanism-based, inhibitors of this enzyme.

Platform BF: Cardiac Muscle II

3932-Plat
Proteomics of the Human Cardiac Intercalated Disc: A More Complex Multi-Functional Structure than was Previously Thought
Cristobal G. dos Remedios[1], Colleen Estigoy[2], Darryl Cameron[3], Joshua W.K. Ho[3], Benjamin Herbert[4], Matthew Padula[4], Russel Pickford[5], Michael Guilhaus[5], Jacob Odeberg[6], Fredrik Ponten[7].
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The intercalated disc (ICD) of cardiac muscle joins one cardiomyocyte to several others. It transmits contractile force between these heart muscle cells, but it also must allow action potentials, ions and even small molecules to cross the junctions, it contains multiple receptors, and must permit the cardiomyocytes to grow. The literature contains 142 proteins in mammalian hearts that were identified using a wide range of techniques. Here we employ two technologies that nearly double this number. We use Fourier transform mass spectrometry to identify 84 proteins based on an analysis of their tryptic peptides using purified (but not pure) ICDs from human left ventricles from four non-failing hearts, only about half of which (43) were previously known. We then explore the Human Proteome Atlas (HPA) database to identify 162 ICD proteins using

its >6000 anti-human polyclonal, affinity-purified antibodies of which 49 were identified by MS and 79 were novel. HPA antibodies were available for 82 the 142 proteins but only 43 reacted positively with the ICDs based on immunohistochemistry. We supplement these approaches with 14 proteins identified using conventional 2-DE. Combining all techniques. Only 14 proteins were common to the MS data, HPA antibodies and the literature. We can categorize all 273 identified ICD proteins according to their known functions and demonstrate their functional interactions in a single inter-active relationship according to their functions: (i) adhesion, anchoring and binding (88); (ii) enzymes (46); (iii) proteins that maintain the structure and function of the ICDs (35); (iv) myofibrillar (34); (v) channels (32); (vi) ligands and their receptors (18); (vii) cytoplasm proteins (6); and (viii) mechanoreceptors (4). We will now extend these analyses to ICD proteins that change as a result of human heart failure.

3933-Plat

Quantitative Analysis of MyBP-C Phosphorylation in Human Heart using Phosphate Affinity SDS-Page

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Phosphorylation sites in Cardiac MyBP-C have been predicted at Ser273,282 and 302 but studies in intact tissue have identified 5 phosphorylted sites and suggested up to 4.6molsPi/mol MyBP-C is present in human heart.

We analysed MyBP-C phosphospecies in human heart myofibrils by phosphate affinity SDS-PAGE using a non-specific antibody raised against the MyBP-C peptide 2-14. We observed six bands corresponding to 0, 1P, 2P, 3P, 4P, 5P phospho-species. Control experiments with pure MyBP-C indicated that the antibody labelled all phosphospecies equally. The assigned phosphorylation levels were confirmed by staining western blots with PhosTools phosphoprotein stain. This separation permits direct quantitative determination of MyBP-C phosphospecies without need for calibration.

In donor heart myofibrils the highly phosphorylated species predominated: 0, $7 \pm 3\%$: 1P, $1 \pm 1\%$: 2P, $23 \pm 7\%$: 3P, $41 \pm 2\%$: 4P, $20 \pm 8\%$ (n=4) from which total phosphorylation of MyBP-C was calculated to be 3.4molsPi/mol. In failing heart unphosphorylated MyBP-C predominated (0, $48 \pm 4\%$: 1P, $4 \pm 4\%$: 2P, $27 \pm 5\%$: $1 \pm 1\%$: 3P, $17 \pm 4\%$: 4P, $4 \pm 2\%$, n=4) and calculated total phosphorylation was 1.5 molsPi/mol. Total phosphorylation in failing heart myofibrils was 44% of donor and in myectomy samples from HCM patients it was 29% of donor, compared with 45 and 40% respectively determined in previous assays.

We conclude that MyBP-C is highly phosphorylated in vivo with significant phosphorylation of at least 5 sites and that phosphorylation is dynamic, being greatly reduced in pathological muscle. Initial tests using antibodies specific to Ser 273, 282 and 302 show distinct patterns on phosphate affinity SDS-PAGE indicating varying preferences for the highly phosphorylated species of MyBP-C in normal and pathological muscle.

3934-Plat

Identification of Amino Acid Residues in the Cardiac Myosin Binding Protein-C Motif Important for Actin Binding

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N-terminal domains of cardiac myosin binding protein-C (cMyBP-C) can activate actomyosin interactions in the absence of Ca^{2+} and bind to actin in a phosphorylation dependent manner. We have previously shown that two N-terminal domains, C1 and the MyBP-C motif (''M'') domain, bind specifically to actin and to thin filaments; however, the sequences or residues that mediate actin binding have not been identified. The goal of this study was to identify residues in the M-domain that contribute to actin binding and to investigate whether interactions between the M-domain and actin mediate the activating properties of cMyBP-C. We therefore used alanine-scanning mutagenesis to target candidate actin binding sites in the M-domain that bear homology to the actin binding motifs in other known actin binding proteins and to assess the effects of mutations on the ability of recombinant proteins to bind actin and activate actomyosin interactions in motility assays. Results demonstrate that mutation of select positively-charged amino acids in the M-domain that are homologous to binding motifs in known actin binding proteins reduced binding of cMyBP-C to actin. The mutations also reduced or eliminated the activating properties of recombinant cMyBP-C in *in vitro* motility assays. However, mutation of other positively-charged amino acids did not affect actin binding or protein functional properties. These results indicate that specific residues within the M-domain confer actin binding and that interactions with actin contribute to the functional effects of recombinant cMyBP-C N-terminal proteins. Supported by NIH HL080367 to SPH and a NSF Graduate Research Fellowship to JFS.

3935-Plat

N-Terminal Fragments of Cardiac Myosin Binding Protein-C Inhibit Actomyosin Motility by Tethering Actin

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Cardiac myosin binding protein-C (cMyBP-C) mutations are a leading cause of hypertrophic cardiomyopathy. cMyBP-C has 11 domains, C0 through C10, that bind sarcomeric proteins, including myosin and actin. A 29 kD N-terminal fragment (C0C1f) of cMyBP-C containing the first two domains C0 and C1 and the first 15 residues of the conserved MyBP-C motif is cleaved from cMyBP-C following ischemic-reperfusion injury (Sadayappan et al., JMCC 44:S44, 2008). Expressed C0C1f fragments inhibit actin velocities in the motility assay at a 2:1 molar ratio to myosin, similar to other N-terminal fragments: C0C3, C0C2, and C1C2. Interestingly, fragments containing only the C0C1 domains do not alter velocity, suggesting the additional 15 residues in C0C1f are necessary for inhibition. Adding C0C1 to the motility assay can partially reverse the C0C3-mediated inhibition of velocity, suggesting C0C1 may compete with C0C3 for actin binding. cMyBP-C fragments may affect motility by creating a tether between actin and the flowcell surface. To test this, motility experiments were performed under high ionic strength, saturating MgATP, and in the absence of methylcellulose, conditions in which most actin filaments diffuse away from the surface due to weak interactions with myosin. In the presence of C0C2, many actin filaments bound and translocated on the surface, confirming this fragment's tethering capacity. Additionally, in the laser trap we adhered C0C3 fragments to a bead in the absence of myosin and observed C0C3 transiently binding to a single actin filament with an ~100 ms attached lifetime. We also saw evidence that C0C3 may partially unfold under load. These experiments strongly suggest that N-terminal domains of cMyBP-C containing the MyBP-C motif tether actin filaments and provide one mechanism for modulating actomyosin motion generation, i.e. by imposing an effective viscous load within the sarcomere.

3936-Plat

PKC Phosphorylation of Titin's PEVK Element–A Novel and Conserved Pathway for Modulating Myocardial Stiffness

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Protein Kinase C (PKC) regulates contractility of cardiac muscle cells by phosphorylating multiple thin- and thick- filament based proteins, and plays key roles in development of cardiovascular pathologies. Myocardial sarcomeres also contain a third myofilament, titin, which we demonstrate here to also be phosphorylated by PKC. Titin phosphorylation was observed in skinned myocardial tissues following incubation with PKCα and this effect was exacerbated ~5 fold in the mouse and ~2.5 fold in the pig by preincubation with Protein Phosphatase 1 (PP1). In vitro phosphorylation of recombinant protein representing titin's spring elements shows that PKCα targets the PEVK spring element. Mass spectrometry in combination with site-directed mutagenesis identified two highly conserved sites in the PEVK region that are phosphorylated by PKCα (S11878 and S12022); when these two sites are mutated to alanine, phosphorylation is effectively abolished. Mechanical experiments with murine and porcine skinned LV myocardium revealed that PKCα significantly increases titin-based passive tension in a sarcomere length (SL)-dependent fashion. Single molecule force-extension curves show that PKCα decreases the PEVK persistence length (from 1.20 nm to 0.55 nm), without altering the contour length, and using a serially-linked wormlike chain (WLC) model we show that this results in an ~20% increase in titin-based passive force with a SL dependence that is similar to that measured in skinned myocardium following PKCα phosphorylation. We conclude that PKC phosphorylation of titin is a novel and conserved pathway that links myocardial signaling and myocardial stiffness.

3937-Plat

Titin Strucure and Extensibility in Healthy and Failing Hearts

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The giant sarcomeric protein, titin is the primary determinant of myocardial passive stiffness. In failing human hearts however, it has been proposed that reduced myofilament passive tension is due to an altered titin isoform expression profile[1, 2]. In this study we set out to directly quantify the tensile strength of titin molecules isolated from healthy and diseased myocardium.